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Evidence for Immune-Mediated Destruction as Mechanism for LCMV-Induced Anemia in Persistently Infected Mice

KATHLEEN A. STELLRECHT-BROOMHALL

ABSTRACT

A docile substrain of lymphocytic choriomeningitis virus (LCMV) causes a persistent infection in adult C3HeB mice and induces a severe anemia, which, unlike the viremia, eventually resolves. Measurements of red blood cell (RBC) survival rates demonstrated an increased rate of RBC clearance in these animals, indicating a hemolytic process for the anemia. Normal clearance rates of RBCs from infected mice transfused into control mice suggested that there was not an intrinsic defect in these cells. It also appeared that RBC destruction was immune-mediated, as cyclophosphamide treatments prevented the onset of anemia in infected mice, whereas adoptive transfer (AT) of immune splenocytes into immunocompromised mice reestablished the condition. The AT experiments also demonstrated that the onset of anemia correlated with the functional state of the immune cells. In addition, opsonization of RBCs was demonstrated by macrophage phagocytosis, and the appearance of opsonized RBCs corresponded with the course of the anemia. These findings support a hypothesis of RBC opsonization and subsequent phagocytosis by macrophages of the reticuloendothelial system as the mechanism for RBC destruction in LCMV-induced hemolytic anemia.

Lymphocytic choriomeningitis virus (LCMV), the prototype of the arenavirus family, is found as a natural infection in mice (29). Studies of this model have shown it to be a "Rosetta stone" for immunopathologist. Burnet's theory of the clonal deletion of self-reactive lymphocytes was based, in part, on what was known about congenital LCMV infections of mice (4). In more recent times, the role of virus-specific cytotoxic T cells (19), as well as their H-2 restriction (41); the mechanisms of immune complex diseases (26); and the demonstration of the ability of viruses to distort cellular functions in the absence of cytotoxicity (25) have all emanated from the murine LCMV model.

Previously, a unique substrain of LCMV (docile) that establishes a persistent infection when inoculated intracranially into adult C3HeB mice was described (17). Hematologic studies demonstrated that these animals experience a severe pancytopenia 2 to 3 weeks postinfection (p.i.), which, unlike the viremia, eventually resolves (3). Bone marrow evaluations indicated that the leukopenia and thrombocytopenia are secondary to loss in cell production. However, the mechanism for the anemia is not as clear. Peripheral blood

Department of Biology, Rensselaer Polytechnic Institute, Troy, New York

smears demonstrating marked red blood cell (RBC) abnormalities suggest a hemolytic process, whereas an erythroid hypoplasia during the first week of infection implies that decreased cell production may also have a role (3). In addition, cyclophosphamide treatment prevents the anemia, suggesting that the underlying mechanism is immune mediated (3).

In the present study, the transient anemia in LCMV-infected mice was more closely associated with a hemolytic process rather than suppression of hematopoiesis. In addition, the role of the immune system in the anemia and the mechanism(s) by which it mediates its effects were evaluated.

METHODS

Mice. Three- to four-week old female C3HeB/FeJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and held for 1 week before use. Care was taken to ensure that all regulations regarding safety, ethics, and use of animals were met in accordance with the guidelines established by the animal safety committee and the National Institutes of Health.

Virus. A triple plaque-purified docile substrain of LCMV was used (18). Virus stocks were grown in MDCK cells for no more than three passages beyond plaque purification. Mice were injected intracranially with 600 plaque-forming units (PFU) of virus.

Hematocrits and plasma free-hemoglobin concentrations. Blood was collected from the retro-orbital plexus into heparinized capillary tubes. Microhematocrit measurements were performed as previously described (3). Plasma free-hemoglobin levels were determined spectrophotometrically by the procedure of Martinek (22). Briefly, capillary tubes were centrifuged for 5 min in a microhematocrit centrifuge (Clay Adams, Parsippany, NJ). The plasma was separated from the cells and diluted 1:10 in isotonic saline, and the absorbency of the diluted serum was measured at 415 and 455 nm in a Beckman Spectrophotometer Model 25 (Beckman Instruments Inc., Irving, CA).

RBC survival studies. Red blood cell survival studies were performed essentially as described by Cox and Keast (9). Briefly, blood collected from the jugular vein was anticoagulated in a 3.8% (wt/vol) sodium citrate solution and washed three times with phosphate-buffered saline (PBS). Packed RBCs were incubated at 37°C for 45 min with ^{51}Cr-sodium chromate (New England Nuclear, Wilmington, DE), at a concentration of 300 μCi/ml of packed cells. The cells were again washed and resuspended to a 30% cell suspension in PBS. Recipient mice were given intravenous (i.v.) injections of 0.2 ml of the cell suspension. Duplicate blood samples were taken from the retro-orbital venous plexus at various times post-transfusion (PT). When all samples were collected, radioactivity was measured in a gamma counter (Nuclear-Chicago, Des Plaines, IL). The initial samples, taken 24 h PT, were used as the baseline (100% survival) from which the percentages of maximum radioactivity were calculated. The RBC half-lifes were calculated according to the procedure of Wollman and colleagues (40). Mean RBC survival times were calculated as a weighted mean in accordance with the method recommended by the Panel on Diagnostic Application of Radioisotopes in Hematology (27).

Histologic examinations. Spleens, lymph nodes, and femurs from four mice 21 days p.i., as well as from aged-matched controls, were weighed, fixed in Formalin, and embedded in paraffin. Thin sections were stained with hematoxylin–eosin. Organ histology was evaluated by Dr. A. Balasubramaniam, pathologist at Ellis Hospital, Schenectady, NY.

Adoptive transfer experiments. Cyclophosphamide (Sigma Chemical Co., St. Louis, MO) was given to recipient mice at a dose of 150 mg/kg by intraperitoneal (i.p.) inoculation 3 days after virus injection (3). The effectiveness of the treatment was demonstrated by loss of anemia and of virus-specific cytotoxic T-cell activity (17). Pooled splenocytes from LCMV-infected mice, passed through stainless steel meshes, were washed and resuspended to 3.5×10^8 cells/ml in Minimal Essential Medium (MEM) with heparin (20 U/ml, Sigma). Cyclophosphamide-treated recipient mice were given i.v. injections with 0.2 ml of the splenocyte suspension on day 5 p.i.

***In vitro* erythroid phagocytosis assay**. The *in vitro* erythroid phagocytosis assay was modified from the procedure of Zupanska et al. (42). Briefly, thioglycolate-elicited peritoneal macrophages were prepared as described (1). A suspension of 5×10^5 cells/ml in MEM-10 (1 ml) was allowed to adhere to glass coverslips in a 24-well Falcon plate (Becton Dickinson Labware, Oxnard, CA) by overnight incubation at 37°C with CO_2

and humidity. Cells from retro-orbital venous blood pooled from four mice were washed with PBS and resuspended to a concentration of 1×10^8 cells/ml in MEM. The medium in each of four wells was replaced with 1 ml of RBC suspension, and the plates were incubated for 3 hours at 37°C. Coverslips were stained with a May-Grunwald–Giemsa stain, and 500 macrophages on each coverslip were evaluated for RBC phagocytosis.

RESULTS

Comparison of RBC survival rates in infected and normal mice. Red blood cell survival was evaluated to determine if RBC hemolysis does indeed occur in LCMV-infected mice. In these experiments, ^{51}Cr-labeled RBCs from either uninfected or infected mice 14 days p.i. were injected into similar recipient animals, and the RBC half-life (T½), or time at which 50% of the cells are removed, was determined. The T½ of normal RBCs injected into normal recipients extrapolated to approximately 16 days (Fig. 1) , which is within the range expected for normal murine RBCs (31). The RBCs from either infected or uninfected mice injected into infected recipients were cleared at a much faster rate (T½ of approximately 6 to 7 days). These results indicate

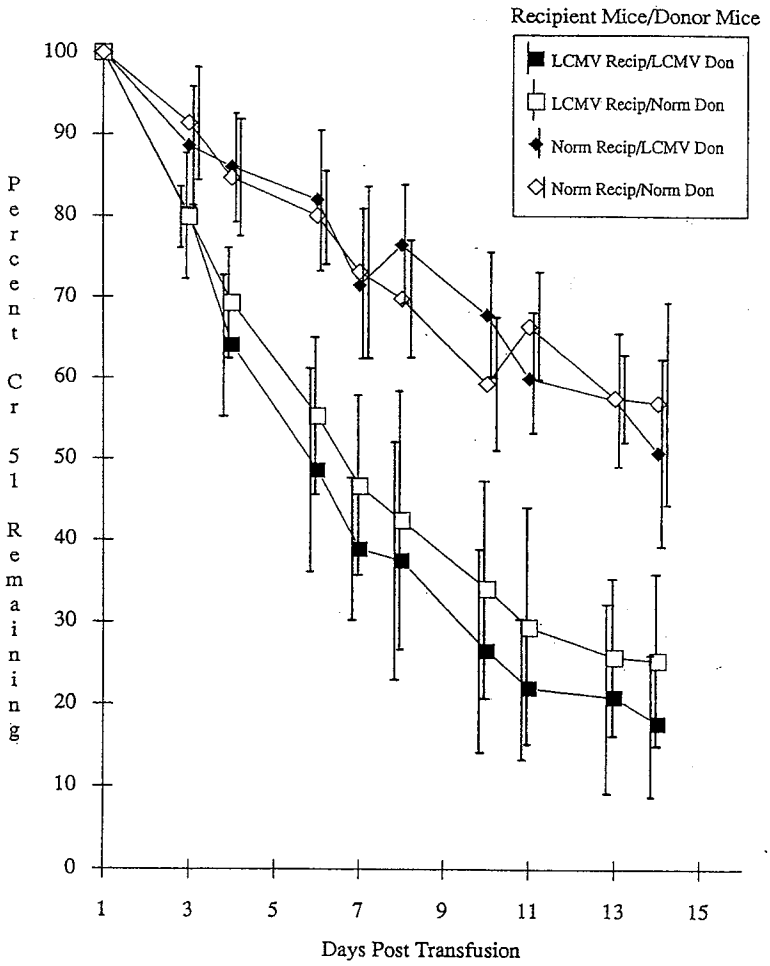

FIG. 1 Survival rates of RBCs as function of both donor and recipient. Sixteen recipients, either uninfected or LCMV-infected 14 days p.i., were tranfused with ^{51}Cr-labeled RBCs from normal or day 14-infected donors. Each point represents mean per cent of maximum radioactivity (radioactivity 1 day PT); error bars represent standard deviations. Results were pooled from four experiments.

that the anemia in LCMV-infected mice was attributable to a destruction or rapid clearance of the RBCs. In addition, RBCs from infected mice injected into normal recipients were cleared at normal rates, suggesting that these cells were basically normal (Fig. 1).

Correlation of RBC survival rates with severity of anemia. The observation that the RBC half-life was significantly shortened in virus-infected mice was consistent with the hypothesis that the anemia in LCMV-infected mice reflects a hemolytic process. However, to demonstrate a cause and effect relation between the accelerated rate of RBC removal and the anemia, it was necessary to demonstrate a correlation between RBC half-lifes and the severity of anemia in the recipient mice. Two methods were chosen to determine if such a correlation existed. In one type of experiment, the RBC survival rates during the course of the infection were compared with the progression of the anemia. In the other type of experiment, the natural variation in the degree of anemia found from one infected mouse to another was exploited.

In the first experimental approach, RBC survival rates were measured at weekly intervals in LCMV-infected mice and age-matched controls. At the time of infection (day 0) and on days 7, 14, 21, and 28 p.i., mice were transfused with normal ^{51}Cr-labeled RBCs, and the radioactivity was measured 24 h later and subsequently every other day for the next 6 days. Because sampling time was reduced from 14 to 7 days, weighted mean survival rates of RBCs, which determine the average cell lifespan, were calculated (27). It was evident that when RBCs were transfused at the time of infection of the recipient, the mean survival time (days) was similar to that of normal mice (24.8 ± 0.5 *v* 25.9 ± 0.9) (Fig. 2). However, if the mice were transfused as early as 7 days p.i., the mean survival time of the RBCs during the ensuing 7 days dropped to 17.6 ± 1.6 *v* 28.6 ± 1.7 days in the control group. The RBC survival time was lowest during the third and fourth weeks of infection, with survival rates of 15.2 ± 2.2 for the 14- to 21-day period and 15.4 ± 0.9 for the 21- to 28-day period (compared with 30.4 ± 2.5 and 27.5 ± 1.8 days in the respective control groups). The RBC survival times then returned to near-normal values during the fifth week of infection (24.4 ± 2.2 *v* 27.4 ± 1.1 days in the control group). It was also evident that the changing survival times of the RBCs reflected the progression and resolution of the anemia as determined by hematocrit readings and that continued suppression of RBC survival during the second, third, and fourth weeks of infection correlated with an exacerbation of the anemia in LCMV-infected mice (Fig. 2).

In the second approach, advantage was taken of the wide variation in the degree of anemia among individual mice. Four mice 14 days p.i. with hematocrits ranging from 22% ot 40% and four age-matched controls were transfused with ^{51}Cr-labeled RBCs. As the data in Figure 3 indicate, the half-lifes of the transfused RBCs reflected the severity of anemia in the recipients: the T½ of the three mice with low hematocrits ranged from 5 to 6.4 days, whereas the T½ in the mouse with the high hematocrit was 8.0 days. It is noteworthy that the T½ of the normal mice again extrapolated to approximately 16 days, twice as long as the T½ in the infected mouse with a normal hematocrit.

Histologic evaluation. The spleens, lymph nodes, and bone marrow from infected mice were evaluated histologically for evidence of viral-associated hemophagocytic syndrome (VAHS). Despite the indications of a hemolytic anemia in LCMV-infected mice, these animals did not appear to have splenomegaly. In fact, the spleens from the infected mice 21 days p.i. were smaller than the spleens from age-matched controls (81.9 ± 16.7 mg *v* 117.2 ± 4.9 mg). Conversely, histologic examinations of the spleens and lymph nodes from infected mice did demonstrate marked follicular and lymphoid hyperplasia, with increases in both the size and the diameter of the follicles. However, there was no effacement of the architecture of the organs, and there were no indications of histiocytic proliferation. Interestingly, there was evidence of hemophagocytosis in the spleen, lymph nodes, and bone marrow of these animals.

Adoptive transfer of hemolytic anemia. Adoptive transfer (AT) experiments were performed to evaluate the role of the immune system in establishing the hemolytic anemia. Initial AT experiments involved the injection of immune splenocytes, pooled from five mice 8 days p.i., into four cyclophosphamide-treated recipient mice 5 days p.i. and following the hematocrits in the recipients (Table 1). Previous reports demonstrated that the hematocrits of cyclophosphamide-treated infected mice remain normal for at least 35 days after treatment (3). Interestingly, the data in Table 1 show that the hematocrits (mean value and standard deviation) of the AT recipients began to decrease on day 6 post-AT and continued to decline until returning to near-normal levels on day 15 post-AT. It was evident from the decreasing hematocrits that anemia was transferred to the recipient mice. In fact, the kinetics of the decreasing hematocrits was similar to that seen

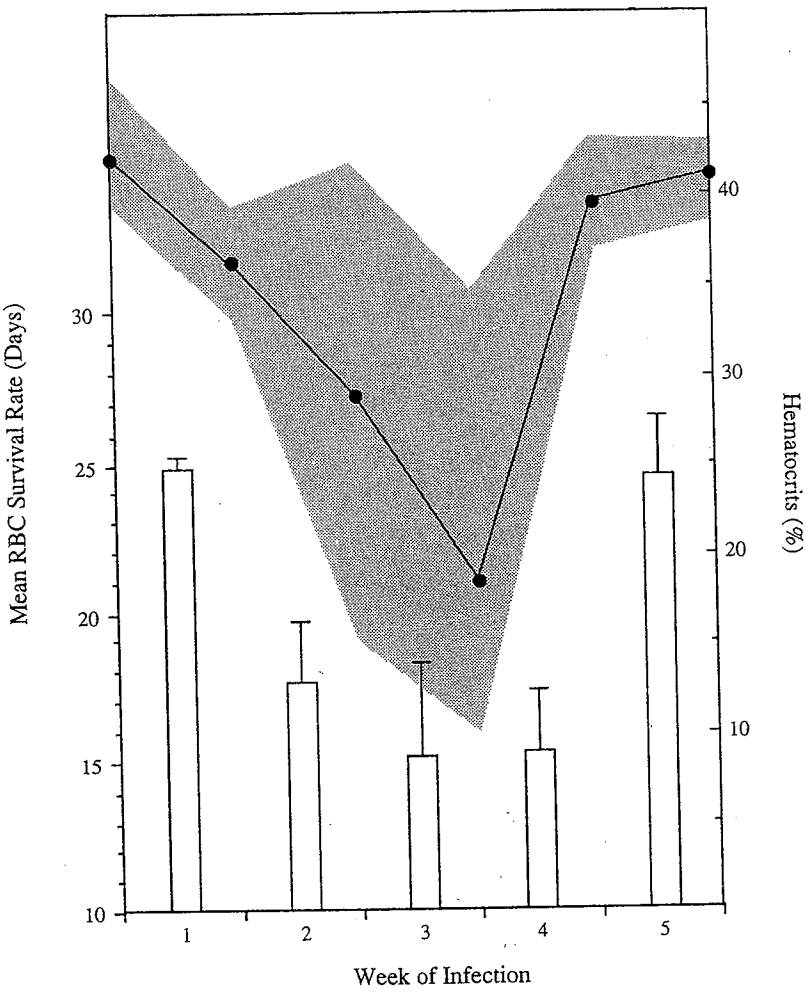

FIG. 2. Survival of RBCs as function of progression of anemia. Groups of five infected or four normal mice were transfused with radiolabeled RBCs on days 0, 7, 14, 21, or 28 p.i., and mean survival rate for each group was calculated from loss of radioactivity during 1-week period. Points connected by lines indicate mean hematocrits for 16 mice at time of transfusion; shaded area represents data range.

during the course of infection except that the anemia resolved itself sooner; i.e., recipients had normal hematocrits by 15 days post-AT, which corresponds to 20 days p.i.

Further experiments demonstrated that the onset of the anemia appeared to be a function of the donor splenocytes (Table 2). In these experiments, splenocytes were pooled from groups of five donor mice at either 5, 8, 11, or 15 days p.i. and injected into four cyclophosphamide-treated recipients 5 days p.i. It was evident that splenocytes from mice 5 days p.i. did not induce anemia in recipient mice until 9 days post-AT, which was day 14 p.i. for both donor and recipient mice. However, splenocytes from mice 11 or 15 days p.i. induced anemia within 3 days of AT even though the recipient mice had been infected for only 8 days. These results support the concept that the onset of anemia was related to the functional state of the immune cells.

Demonstration of RBC opsonization. Although immune-mediated destruction of RBCs is classically attributable to humoral immunity, initial attempts to demonstrate antibody opsonization by the direct antiglobulin test (DAT) were consistently negative (3). However, the DAT is not very sensitive; hence, an *in vitro* erythroid phagocytosis assay was used to increase sensitivity (14). Thioglycolate-elicited macrophages adhering to glass coverslips were incubated with RBCs from normal or infected mice 21 days p.i. and stained with May-Grunwald–Giesma stain to examine phagocytosis. The data in Figure 4 demonstrate the percentage

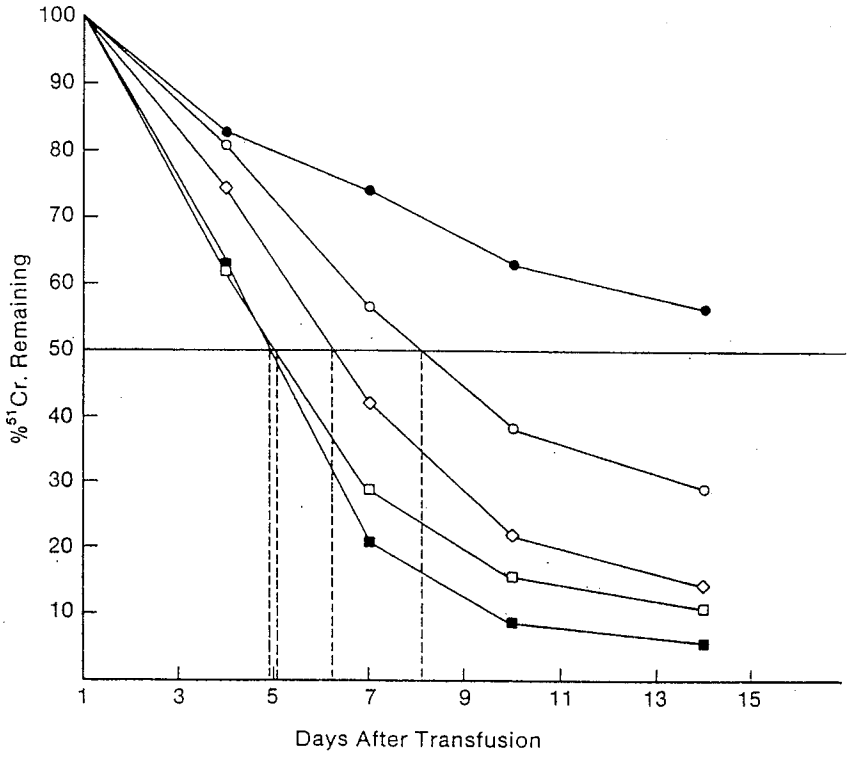

FIG. 3. Survival rates of RBCs reflected individual variation of anemia. Each point represents average per cent of maximum radioactivity (radioactivity 1 day PT) calculated from duplicate samples taken from individual infected recipients transfused 14 days p.i. Recipients were selected on basis of hematocrit (0, 40%, < >, 25%, □, 27%, ■, 22%) at time of transfusion. Seven days later, hematocrits in these mice were 42%, 27%, 18%, and 25%, respectively. Solid point ● represents mean per cent of maximum radioactivity in four uninfected recipient mice.

of macrophages that phagocytized one, two, three, four, or five or more RBCs. A greater percentage of macrophages had phagocytized RBCs from mice 21 days p.i. than had taken up RBCs from uninfected mice (47.9% *v* 14.9%). Even more significant was the total number of RBCs phagocytized. An average of 81.3 RBCs from mice 21 days p.i. compared with an average of 20.6 RBCs from uninfected mice were phagocytized by 100 macrophages. These finding indicated that RBCs from infected mice were opsonized.

In addition, this assay was utilized to correlate the occurrence of sensitized cells with the course of the anemia. In these experiments, blood samples were taken at weekly intervals from LCMV-infected mice and evaluated for the number of macrophages (out of 500) phagocytizing RBCs and the total number of RBCs phagocytized. The data in Figure 5 demonstrate a slight increase in the number of RBCs phagocytized from mice 7, 28, or 35 days p.i. However, RBCs from mice 14 and 21 days p.i. were readily phagocytized, indicating that the occurrence of opsonized RBCs correlated with the anemia.

Plasma free-hemoglobin concentrations. It appeared that RBC destruction was the main cause of anemia in infected mice. To determine if overt intravascular hemolysis was occurring, the plasma free-hemoglobin

TABLE 1. ADOPTIVE TRANSFER OF HEMOLYTIC ANEMIA

	Days Post-AT				
	3	*6*	*9*	*12*	*15*
Hematocrit	42.3	34.3	27.8	30.3	39.2
SD	1.0	3.5	1.4	2.2	5.3

TABLE 2. ONSET OF HA AS FUNCTION OF DONOR SPLENOCYTES

	Days Post-AT		
	3	*6*	*9*
Day 5 donors (SD)	40.5 (1.7)	37.8 (2.5)	30.7 (1.8)
Day 8 donors (SD)	42.3 (1.0)	30.3 (1.4)	27.8 (2.2)
Day 11 donors (SD)	31.8 (2.2)	34.3 (3.5)	40.0 (2.3)
Day 15 donors (SD)	34.0 (3.6)	38.7 (1.9)	45.3 (1.5)
Days p.i. recip.	8	11	14

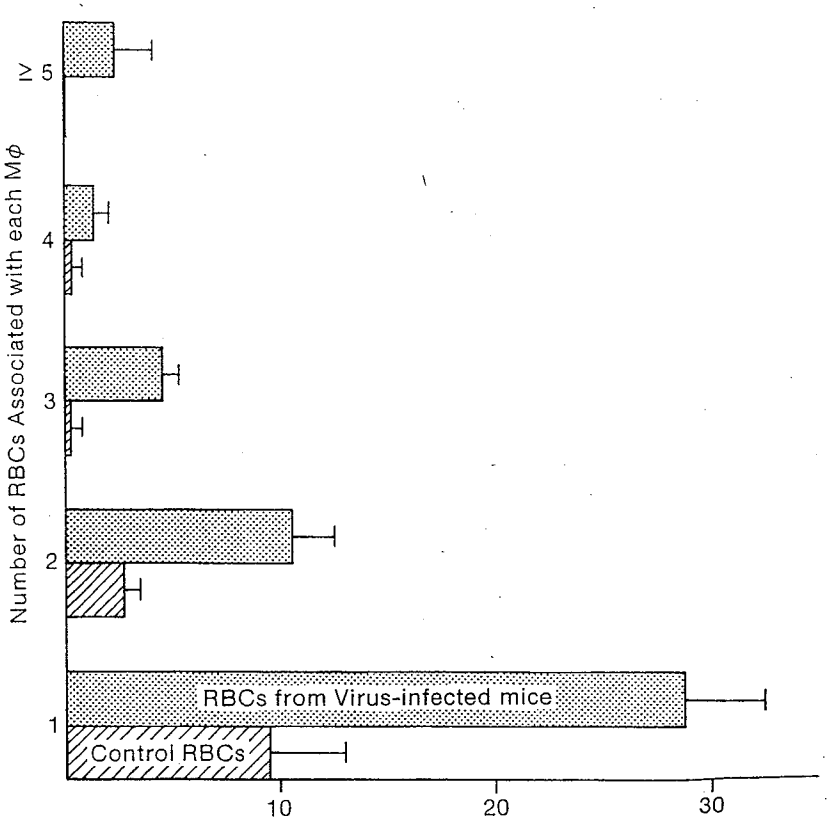

FIG. 4 Degree of *in vitro* erythrophagocytosis as function of RBC source. Bars represent mean percent of macrophages that phagocytized either one, two, three, four, or five or more RBCs. Values represent mean and standard deviation calculated from evaluating 500 macrophages (Mϕ) on each of four coverslips incubated with either control RBCs or RBCs from mice 21 days p.i.

concentrations of infected mice were compared with those of uninfected mice by spectrophotometric determinations. These data are shown in Table 3. These results demonstrated that the plasma free-hemoglobin concentrations of the infected mice were within the normal range.

DISCUSSION

Previously, a report of the hematologic abnormalities in C3HeB mice infected with a docile substrain of LCMV described a transient pancytopenia but was inconclusive as to the cause of the severe anemia occurring

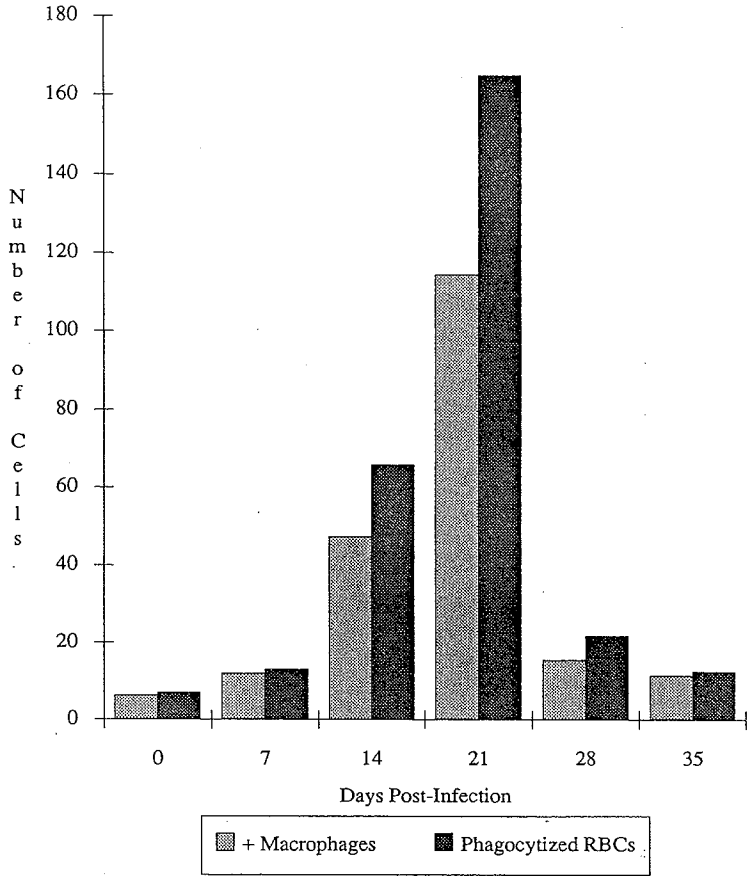

FIG. 5. Appearance of opsonized RBCs during course of LCMV infection. Peritoneal macrophages adhering to coverslips were incubated with RBCs from control mice (0 days p.i.) or from infected mice 7, 14, 21, 28, or 35 days p.i. On each of four coverslips, 500 macrophages were evaluated for number phagocytizing RBCs (+ macrophages) as well as total number of RBCs phagocytized. Bars represent mean values.

TABLE 3. MEAN (SD) PLASMA FREE-HEMOGLOBIN CONCENTRATION IN INFECTED AND CONTROL MICE

Mice	*Hb*	*Hct*
Day 14 p.i.	25.53 (2.69)	32.1 (5.8)
Day 21 p.i.	21.32 (3.80)	21.8 (4.2)
Control	26.45 (6.26)	43.1 (1.0)

from the second to the fourth week of infection (3). Normally, homeostasis of blood components is maintained by a balance between production and destruction, and cytopenia occurs when this balance is disturbed. Bone marrow examinations had indicated that the leukopenia and thrombocytopenia are secondary to a production defect, but the same is not true for the anemia (3). It is clear that during the first week of infection, there is an erythroid hypoplasia. However, normal numbers of erythroid cells are reattained by day 11 p.i., and a continuing compensatory response results in an erythrocyte precursor number twice that of normal values by

21 days (3). Nonetheless, the number of RBCs in the circulation decreases by the same factor during this period, suggesting that suppression of erythropoiesis is an unlikely mechanism for the anemia.

Abnormal RBC morphology; i.e., spherocytes and schistocytes, seen on peripheral blood smears from mice 14 and 21 days p.i. suggests that the anemia is hemolytic (3). Measurements of RBC survival rates in the present report clearly indicated that these cells were cleared faster in infected mice than in normal mice, implying a hemolytic process for the anemia (see Fig. 1). Furthermore, it was demonstrated that the anemia reflected the accelerated rate of RBC clearance and not the erythroid hypoplasia. The evidence for this association came from several findings. First, at the height of anemia, RBCs were cleared at least twice as fast in infected as in normal mice (see Fig. 1). Second, the changes in the mean RBC survival rate during the course of the disease reflected the progression and resolution of the anemia (see Fig. 2). Third, the rate of RBC destruction varied with the severity of the anemia in virus-infected recipients (see Fig. 3).

Brö–Jorgensen and Knudtzon (2) also evaluated hematologic abnormalities in LCMV-infected mice. Although their model involved an abortive immunizing infection rather than persistence, they, too, demonstrated a marked suppression in hematopoiesis during the first week of infection. However, this suppression results in only a modest anemia 10 days p.i., supporting the hypothesis that the erythroid hypoplasia in persistently infected mice has a minor role in the severe anemia occurring 21 days p.i.

Based on the RBC survival studies, it is not unrealistic to assume that the anemia in virus-infected mice is the result of an accelerated rate of destruction. Alternately, in light of a severe thrombocytopenia, the removal of RBCs from the circulation could be secondary to bleeding. Indeed, a pronounced hemorrhagic diathesis is associated with certain human arenavirus infections (28), and the docile virus causes severe bleeding into the thoracic cavity of lethally infected SWR/J mice (11). However, hemorrhaging has never been observed in virus-infected C3HeB mice.

Histologic examinations of lymphoid tissue from infected mice indicated that the RBC destruction was not the result of VAHS. This condition is defined as a benign histiocytic proliferation of hemopoietic and lymphoid tissues with marked hemophagocytosis in association with a chronic viral infection (36). Although examinations of spleen and bone marrow from infected mice demonstrated hemophagocytosis, it was not as marked as that typically seen in VAHS, and there were no indications of histiocyte proliferation. In addition, the appearance of a marked erythroid hyperplasia contradicts any indication of VAHS.

It is known that cyclophosphamide treatment negates the anemia in LCMV-infected mice, suggesting that the anemia is immune mediated (3). However, a more definitive display of the role of the immune system is the transfer of anemia with immune splenocytes (see Table 1). The AT experiments also indicated that the anemia was a function of the immune splenocytes by demonstrating that the appearance of the anemia in the recipient mice correlated with the course of LCMV infection in the donor mice (see Table 2). Unfortunately, evaluations of the type of cell (e.g., T or B lymphocytes) required for disease transfer were inconclusive. It appeared that AT of splenocytes depleted of IgG^+ and IgM^+ cells or Thy 1^+ cells through antibody–complement-mediated lysis were still capable of establishing the anemia (unpublished data). However, because plasma cells do not present surface Ig, it is probably that these cells were transferred with either splenocyte population (7).

Evidence for the role of the humoral immunity in mediating RBC destruction comes through the demonstration of RBC opsonization by *in vitro* erythroid phagocytosis (see Fig. 4). This highly sensitive assay is based on the concept that opsonized RBCs, binding to macrophage via Fc receptors, trigger phagocytosis (14). The fact that RBCs from LCMV-infected mice are susceptible to phagocytosis indicates that these cells are opsonized (see Fig. 4). In addition, the appearance of opsonized RBCs was correlated with the course of the anemia, suggesting a role for immune-mediated RBC destruction in LCMV-induced hemolytic anemia (see Fig. 5). Recently, it has been reported that antibodies can be eluted from the surface of RBCs from LCMV-infected mice and have been determined to be IgG_{2a} immunoglobulins (37). Furthermore, the demonstration, by ELISA, that antibodies eluted from the red cells recognize RBC antigens but not LCMV antigens (37) indicates that this anemia is actually autoimmune.

The *in vitro* evidence of RBC opsonization, however, appears to contradict the *in vivo* RBC survival studies, which suggest that RBCs from infected mice are normal. This can be explained by the presence of an abundance of unopsonized, unlabeled epitopes in the recipient mice that compete for the antibodies. This

scenario is even more plausible if the epitopes are common to many cells. Indeed, serum from LCMV-infected mice is incapable of inducing anemia upon transfusion into normal mice and only weakly opsonizes normal RBCs for macrophage phagocytosis (unpublished results), suggesting most of the antibody in infected mice is bound rather than free in the serum.

Antibody-mediated RBC destruction can occur in many forms, such as complement-mediated lysis, antibody-dependent cellular cytotoxicity (ADCC), and phagocytosis via Fc receptor binding. The mode of destruction usually is directed by the antibody populations involved, although the cell-surface antigen may also be instrumental. Immunoglobulin isotypes and IgG subgroups differ in their abilities to bind complement and in their affinity for Fc receptors (42). It is evident that the erythroid phagocytosis assay not only demonstrates the presence of an opsin on the RBC surface, but it also illustrates the ability of the opsin to mediate phagocytosis (23). This finding suggests a potential mechanism for RBC destruction *in vivo;* i.e., RBCs in LCMV-infected mice are opsonized by antibodies and then phagocytized by the reticuloendothelial system. This hypothesis is further supported by the evidence of hemophagocytosis in the spleen and lymph nodes and by the presence of spherocytes in the peripheral blood smears, which result from partial or incomplete phagocytosis of opsonized RBCs (21). Furthermore, the normal plasma free-hemoglobin concentrations in LCMV-infected mice indicated that the hemolytic anemia is not secondary to mechanisms that result in overt intravascular destruction, such as ADCC and complement-mediated lysis (20).

The antigen to which the opsonizing antibody is directed is yet to be elucidated. Numerous epitopes on the RBC surface represent potential candidates, and because LCMV-infected mice also experience leukopenia and thrombocytopenia concurrent with the anemia (3), it is possible that the antigen is common to all hemopoietic cells. It is evident that the immune-mediated destruction of the RBCs is not the result of an immune response directed against viral particles bound to RBC membranes. Plaque assays of washed RBCs, as well as indirect immunofluorescence for LCMV antigens, were negative (3), indicating that LCMV does not associate with the RBCs. Furthermore, normal survival rates of RBCs from infected mice suggest that the cells are normal and not in direct association with the virus.

It is interesting that although LCMV infection of these animals is lifelong (7), the anemia is transient. This finding may reflect a suppressive mechanism being activated. Indeed, Cox and McAuliffe (10) have demonstrated that the anti-mouse RBC antibody produced on transfusion of rat RBCs results in the induction of suppressor cells specific for anti-mouse RBC antibodies. However, the involvement of immune suppression in the disappearance of LCMV-mediated hemolytic anemia has yet to be demonstrated.

Autoimmune hemolytic anemia (AIHA) is a rare, often severe, complication of many viral infections that plague man (32), with the most recent example being acquired immunodeficiency syndrome (5, 33, 34). Although this problem has been recognized for decades, little is known about the mechanisms by which viruses induce this immune-mediated destruction. Experimentally, it has been difficult to study virus-induced AIHA because a practical virus–animal model has been lacking. Many retrovirus infections of animals, particularly equine infectious anemia, are associated with an immune-mediated hemolytic anemia. However, with these infections, RBC destruction is actually the result of antiviral antibodies directed against viral particles bound to the RBCs and not an aberrant immune response (6, 16, 35, 38–40).

Our understanding of how viruses induce AIHA is in a primitive state. The development of autoimmunity has been associated with polyclonal B-cell activation (3, 8,15), loss of suppression of anti-erythrocyte B cells (10), or crossreacting antibodies elicited by molecular mimicry of the viral antigens (12) or by the primary antibody response against the virus; i.e., an anti-idiotype antibody (24). It is hoped that the AIHA seen in C3HeB mice infected with LCMV will be a suitable model to test these possibilities.

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REFERENCES

1. Argyris, B.F. 1981. Adoptive transfer of macrophages. pp. 493–500. In H.B. Herscowitz, H.T. Holden, J.A. Bellanti, and A. Ghaffer, eds: Manual of Macrophage Methodology. Marcel Dekker, New York.
2. Brö–Jorgensen, K., and S. Knudtzon. 1977. Changes in hemopoiesis during the course of acute LCM infection in mice. Blood 49:47–57.
3. Broomhall, K.S., M. Morin, D.C. Pevear, and C.J. Pfau. 1987. Severe and transient pancytopenia associated with a chronic arenavirus infection. J. Exp. Pathol. 3:259–269.
4. Burnet, F.M. 1957. A modification of Jerne's theory of antibody production using the concept of clonal selection. Aust. J. Sci. 20:67–69
5. Chang, M-C., C-C. Shin, M.L. Chu, and W-C. Huang. 1986. Acquired immunodeficiency syndrome (AIDS) related complex. J. Formosan Med. Assoc. 85:890–897.
6. Cheevers, W.P., and T.C. McGuire. 1985. Equine infectious anemia virus: immunopathogenesis and persistence. Rev. Infect. Dis. 7:83–88.
7. Cooper, M.D., J. Kearney, and I. Scher. 1984. B lymphocytes. pp. 43–55. In W.E. Paul, ed: Fundamental Immunology. Raven Press, New York.
8. Coutelier, J.P., J.T.M. van der Logt, F.W.A. Heessen, A. Vink, and J. van Snick. 1988. Virally induced modulation of murine IgG antibody subclasses. J. Exp. Med. 168:2372–2378.
9. Cox, K.O., and D. Keast. Rauscher virus infection, erythrocyte clearance studies and autoimmune phenomenon. J. Natl. Cancer Inst. 50:941–946.
10. Cox, K.O., and A. McAuliffe. 1983. Experimental erythrocyte autoantibodies V: induction and suppression of red blood cell autoantibodies in mice injected with rat bromelain-treated red blood cells. Cell. Immunol. 81:22–27.
11. Eyler, Y.L., C.J. Pfau, K.S. Broomhall, and A.R. Thomsen. 1989. The combination of MHC and non-MHC genes influence murine LCM virus pathogenesis. Scand. J.Immunol. 29:527–533.
12. Fujinami, R.S., and M.B.A. Oldstone. 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. Science 230:1043–1045.
13. Garzelli, C.F., R.E. Taub, J.E. Scharff, B.S. Prabhakar, F. Ginsberg–Fellner, and A.L. Notkins. 1984. Epstein-Barr virus transformed lymphocytes produce monoclonal autoantibodies that react with antigens in multiple organs. J. Virol. 52:722–725.
14. Griffin, F.M., J.A. Griffin, J.E. Leider, and S.C. Silverstein. 1975. Studies of the mechanism of phagocytosis I: requirements for circumferential attachment of particle-bound ligands to specific receptors of the macrophage plasma membrane. J. Exp. Med. 142:1263–1282.
15. Hang, L., J.H. Slack, C. Amundson, S. Izui, A.N. Theofilopoulos, and F.J. Dixon. 1983. Induction of murine autoimmune disease by chronic polyclonal B-cell activation. J. Exp. Med. 157:874–883.
16. Halliwell, R.E. 1982. Autoimmune diseases in domestic animals. JAVMA 181:1088–1096.
17. Jacobsen, S., R.M. Friedman, and C.J. Pfau. 1981. Interferon induction by lymphocytic choriomeningitis virus correlates with maximum virulence. J. Gen. Virol. 57:275–283.
18. Jacobsen, S., and C.J. Pfau. 1980. Viral pathogenesis and resistance to defective interfering particles. Nature (London) 283:311–313.
19. Lundstedt, C. 1969. Interaction between antigenically different cells. Acta Pathol. Microbiol. Scand. 75:139–152.
20. Leavell, B.S., and O.A. Thorup. 1976. Fundamentals of Clinical Hematology. 4th ed. WB Saunders, Philadelphia.
21. LoBuglio, A.F., R.S. Contran, and J.H. Jandl. 1967. Red cells coated with immunoglobin G: binding and sphering by mononuclear cells in man. Science 158:1582–1585.
22. Martinek, R.G. 1965. Spectrophotometric determination of haemoglobin in serum or plasma. Proc. Assoc. Clin. Biochem. 3:228–232.

23. Nance, S.J., P. Arndt, and G. Garratty. 1987. Predicting the clinical significance of red cell alloantibodies using a monocyte monolayer assay. Transfusion 27:449–452.

24. Nepom, J.T., H.L. Weiner, M.A. Dichter, M. Tardieu, D.R. Spriggs, C.F. Gramm, M.L. Powers, B.N. Fields, and M.I. Greene. 1982. Identification of a hemagglutinin specific idiotype associated with reovirus recognition shared by lymphoid and neural cells. J. Exp. Med. 155:155–167.

25. Oldstone, M.B.A., P. Blount, A. Tishon, M. Rodriguez, R. von Wedel, and P.W. Lampert. 1982. Virus-induced alterations in homeostasis: alterations in differentiated functions in infected cells *in vivo*. Science 218:1125–1127.

26. Oldstone, M.B.A., and F.J. Dixon. 1969. Pathogenesis of chronic disease associated with persistent lymphocytic choriomeningitis viral infection. J. Exp. Med. 129:483–505.

27. Panel of Diagnostic Applications of Radioisotopes in Hematology. 1977. Recommended method for radioisotope platelet survival studies. Blood 50:1137–1144.

28. Peters, C.J. 1984. Arenaviruses. pp. 513–545. In R. Belshe, ed: Textbook of Human Virology. PSG Publishing, Littleton, MA.

29. Pfau, C.J., G.H. Bergold, J. Casals, K.M. Johnson, F.A. Murphy, I.R. Pederson, W.E. Rawls, W.P. Rowe, P.A. Webb, and M.C. Weissenbacher. 1974. Arenaviruses. Intervirology 4:207–213.

30. Pfau, C.J., M.F. Saron, and D.C. Pevear. 1985. Lack of correlation between cytotoxic T cells and lethal murine lymphocytic choriomeningitis. J. Immunol. 135:597–602.

31. Pinkerton, P.H., and R.M. Bannerman. 1968. The hereditary anemias of mice. Hematol. Rev. 1:120–181.

32. Pirofsky, B. 1969. Autoimmunization and the autoimmune hemolytic anemias. William & Wilkins, Baltimore.

33. Puppo, F., A. Torresin, G. Lotti, E. Balleari, G. Orlando, and F. Indiveri. 1988. Autoimmune hemolytic anemia and human immunodeficiency virus (HIV) infecion. Ann. Intern. Med. 109:249–250.

34. Rapoport, A.P., J.M. Rowe, and A. McMican. 1988. Life threatening autoimmune hemolytic anemia in patients with the acquired immune deficiency syndrome. Transfusion 28:190–191.

35. Reilly, C.A., and G.T. Schloss. 1971. The erythrocyte as virus carrier in friend and Rauscher virus leukemias. Cancer Res. 31:841–846.

36. Risdall, R.J., R.W. McKenna, M.E. Nesbit, W. Krivit, H.H. Balfour, R.L. Simmons, and R.D. Brunning. 1979. Virus-associated hemophagocytic syndrome: a benign histiocytic proliferation distinct from malignant histiocytosis. Cancer 44:993–1002.

37. Vella, A.T., and C.J. Pfau. 1991. The characterization of autoantibodies eluted from RBCs of LCMV-infected mice. Autoimmunity. 9:819–830.

38. Werner, L.L., and N.T. Gorman. 1984. Immune-mediated disorders of cats. Symp. Advanced Feline Med 14:1039–1064.

39. Witter, R., H. Frank, V. Moening, G. Hunsmann, J. Lange, and W. Schafer. 1973. Properties of mouse leukemia viruses IV: hemagglutination assay and characterization of hemagglutinating surface components. Virology 54:330–345.

40. Wollmann, R.L., E.J. Pang, A.E. Evans, and W.H. Kirsten. 1979. Virus-induced hemolytic anemia in mice. Cancer Res. 30:1003–1010.

41. Zinkernagel, R.M., and P.C. Doherty. 1974. Restriction of *in vitro* T-cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogenic system. Nature (London) 248:701–702.

42. Zupanska, B., E. Thompson, E. Brojer, and A.H. Merry. 1987. Phagocytosis of erythrocytes sensitized with known amounts of IgG1 and IgG3 anti-Rh antibodies. Vox Sang. 53:96–101.

Address reprint requests to:
Kathleen A. Stellrecht, PhD
Dept. of Clinical Microbiology
Mail Stop A-22
Albany Medical Center Hospital
43 New Scotland Avenue
Albany, NY 12208